LXI. THE OXIDATION OF *l*-ASCORBIC ACID BY PLANT ENZYMES

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That ascorbic acid can be dehydrogenated by an aerobic oxidase present in the apple has been shown by one of us [Zilva, 1934]. It was further found that this enzyme is also capable of dehydrogenating another member of the ascorbic acid series, namely *d*-gluco-ascorbic acid (*d*-3-ketoglucoheptonofuranolactone), which does not possess antiscorbutic activity even in high doses [Zilva, 1935; 1936]. Stereochemical structure and biological activity of these compounds are therefore not determining factors in this enzymic oxidation.

The apple, however, is not the only plant containing an aerobic enzyme which oxidizes ascorbic acid reversibly. Szent-Györgyi [1930; 1931] established the presence of one in cabbage (*Brassica oleracea* L.), Tauber *et al.* [1935] in Hubbard squash (*Cucurbita maxima*), Hopkins & Morgan [1936] in cauliflower, Srinivasan [1936] in the pods of the drumstick tree [*Moringa pterygosperma*), Barron *et al.* [1936] in lettuce, peach and watercress, and Kertesz *et al.* [1936] in pumpkin, pea, string bean, Lima bean, sweet corn, Swiss chard, carrots, parsnips and spinach.

The present investigation deals with a comparative study of the enzymic reversible oxidation of ascorbic acid by the cabbage, cauliflower, cucumber, marrow, apple and potato. All the experiments were carried out under standardized conditions in order to ascertain whether the enzymes or systems of enzymes involved in the oxidation are identical.

EXPERIMENTAL

The enzymic oxidation of ascorbic acid by the cabbage

In some of these experiments the juice of the cabbage was employed, whilst in others the enzyme prepared according to the procedure described by Szent-Györgyi [1931] was utilized.

Preparation of the juice

The juice was obtained by pressing between porcelain plates in a hand-press the leaves (without ribs) previously frozen at $-20°$. Yield about 70 ml. from 100 g. of tissue. A sample of the fresh yellow juice was always adjusted to *p*H 7·6–8·0 and if a precipitate was formed the remainder of the juice was treated in this way and readjusted to the original *p*H 6·0 after removing the precipitate. The juices retained their activity for several weeks on storage in the cold room.

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Preparation of enzyme

The enzyme was precipitated from the freshly expressed juice by saturation with ammonium sulphate after removing impurities with barium acetate (2 ml. of *M* $Ba(CH_3COO)_2$ per 100 ml. of juice) and the excess of the latter reagent by the addition of 5 ml. of saturated ammonium sulphate. The $(NH_4)_2SO_4$-washed precipitate from 100 ml. of juice was dissolved in 50 ml. of *M*/15 KH_2PO_4 or *M*/5 sodium acetate (*p*H 6·0). This clear solution possessed about half the total potency of the original juice and retained its activity without significant loss for long periods on storage in the cold room. It may be added here that the treatment of cabbage juice with alcohol or acetone yielded precipitates of low activity.

*p*H *range of activity and sensitivity to cyanide*

The activity of the enzyme was studied at *p*H 3·0, 5·0, 7·0 and 9·0 in the absence and also in the presence of *M*/1000 NaCN. The test solutions in each case consisted of 3 ml. of ascorbic acid in *M*/5 sodium acetate solution, 9 ml. of buffer solution and 3 ml. of juice which were added in this order. The component solutions were adjusted to the requisite *p*H before mixing. The final concentration of ascorbic acid in the test solution was 2·5 mg. per 15 ml. The controls contained the same amount of ascorbic acid in 15 ml. of *M*/5 sodium acetate. Glass-distilled water was used throughout these and all the following experiments. Immediately after preparation four 3 ml. samples of each solution were delivered into a series of test-tubes, the first one of which contained 0·5 ml. of glacial acetic acid in order to stop the action of the enzyme. The latter was titrated at once with 1·14 *N*/1000 indophenol which thus gave the concentration of ascorbic acid at the beginning of the experiment. The remaining tubes were in turn similarly acidified and titrated after definite intervals.

The results of this experiment, which are given in Table I, show that the juice possessed a range of activity beginning at *p*H 3·0, rising rapidly to a wide

Table I. *Activity and sensitivity to cyanide of the cabbage enzyme*

Time in hours	mg. ascorbic acid in 3·0 ml. of test solution							
	Juice	Juice + *M*/1000 cyanide	Control	Control + *M*/1000 cyanide	Juice	Juice + *M*/1000 cyanide	Control	Control + *M*/1000 cyanide
	Ascorbic acid solutions at *p*H 3·0				Ascorbic acid solutions at *p*H 5·0			
0	0·52	0·51	0·52	0·51	0·51	0·51	0·51	0·52
1	0·47	0·47	0·51	0·51	0·12	0·21	0·51	0·52
2	0·38	0·39	0·51	0·51	0·00	0·10	0·51	0·52
3	0·31	0·32	0·51	0·51	0·00	0·00	0·51	0·52
	Ascorbic acid solutions at *p*H 7·0				Ascorbic acid solutions at *p*H 9·0			
0	0·51	0·51	0·51	0·52	0·50	0·50	0·50	0·50
1	0·19	0·37	0·51	0·52	0·21	0·35	0·50	0·43
2	0·04	0·27	0·51	0·52	0·10	0·21	0·50	0·38
3	0·00	0·17	0·51	0·52	0·03	0·11	0·50	0·36

optimum zone between *p*H 5·0 and 7·0 and falling slowly to *p*H 9·0. The activity curve is thus not symmetrical about the optimum point. Sodium cyanide in a concentration of *M*/1000 is seen to exert an inhibiting action which is greatest near neutrality.

The aerobic nature of the enzymic activity

That methylene blue cannot act as a hydrogen acceptor in place of molecular oxygen in this enzymic oxidation was shown by the fact that in test-tubes containing 5 ml. of juice (*p*H 6·0), 3 mg. of ascorbic acid in 3 ml. of *M*/5 sodium acetate (*p*H 6·0) and 1 ml. of *M*/1000 methylene blue, kept in the dark in evacuated desiccators for 18 hours, the dye was not decolorized.

Action of the enzyme on mono- and di-hydricphenols

(*a*) *Juice.* The technique developed by Pugh & Raper [1927] in their studies of tyrosinase was employed in these experiments. Phenol and catechol were chosen as substrates. The test solutions were prepared by mixing (i) 10 ml. of juice, 2 ml. 4*M*/10 aniline and 2 ml. 2*M*/10 phenol in *M*/5 sodium acetate, and (ii) 10 ml. of juice, 1 ml. 4*M*/10 aniline and 1 ml. 2*M*/10 catechol also in *M*/5 sodium acetate. Both series were diluted to 20 ml. with *M*/5 sodium acetate. Blank tests were carried out with solutions containing similar quantities of aniline, phenol and catechol in 20 ml. of *M*/5 sodium acetate. All the tests were performed at *p*H 6·0 in 100 ml. unstoppered conical flasks. The juice appeared to have no action upon phenol even after standing overnight. The test solutions which contained catechol, however, rapidly developed a red colour and soon afterwards deposited a precipitate consisting of fine red needles (which were identified as dianilinobenzoquinone). This reaction was considered as evidence that the juice was capable of oxidizing catechol to *o*-benzoquinone.

(*b*) *Ammonium sulphate precipitate.* In similar tests in which a solution of the ammonium sulphate precipitate was used in place of the juice, very little action on catechol was occasionally observed. In these cases a red colour developed gradually only after about 2 hours and very little of the red crystalline precipitate separated later. The slight activity in such instances was most probably due to the precipitation with the enzyme of traces of an auxiliary substance present in the juice.

Action of the enzyme on ascorbic acid in presence of catechol

In view of the fact that cabbage juice can oxidize catechol to quinone and that quinones are known to oxidize ascorbic acid rapidly [Zilva, 1928; 1929; 1930; Johnson & Zilva, 1932], it was of interest to ascertain whether the addition of catechol to the juice would increase the rate at which it oxidized ascorbic acid. The actions of the enzyme as present in the juice and of the enzyme precipitated from the juice with $(NH_4)_2SO_4$ were therefore studied on ascorbic acid alone and on ascorbic acid in the presence of catechol and of aniline separately, and of catechol and aniline together. The test solutions were made up to contain 4 mg. of ascorbic acid (in 3 ml. of *M*/15 KH_2PO_4) and 3 ml. of juice or of precipitated enzyme solution. The catechol and aniline were added as 3 ml. of *M*/20 and *M*/10 solutions respectively in *M*/15 KH_2PO_4. The final volumes were in each case made up to 15 ml. with *M*/15 KH_2PO_4. Corresponding control solutions contained buffer in place of enzyme solution. The reactions were studied at *p*H 6·0. Immediately on preparation, four 2·0 ml. samples of each solution were pipetted into a series of test-tubes, the first of which in each series contained 0·5 ml. of glacial acetic acid to arrest the action of the enzyme. After definite intervals the remaining tubes were similarly acidified and titrated with indophenol. The results are given in Table II.

The first point to note is that the precipitated enzyme, which, unlike the juice, was found in the previous experiment to be unable to oxidize catechol,

Table II. *Influence of catechol on the activity of the cabbage enzyme at* pH *6·0*

All these tests were made in presence of $M/15$ KH_2PO_4

mg. ascorbic acid in 2·0 ml. of test solution

Time in hours	Control ascorbic acid solutions				Ascorbic acid solutions containing juice				Ascorbic acid solutions containing precipitated enzyme			
		$M/100$ catechol	$M/50$ aniline	$M/100$ catechol and $M/50$ aniline		$M/100$ catechol	$M/50$ aniline	$M/100$ catechol and $M/50$ aniline		$M/100$ catechol	$M/50$ aniline	$M/100$ catechol and $M/50$ aniline
0	0·50	0·52	0·52	0·52	0·50	0·52	0·51	0·52	0·51	0·51	0·51	0·51
1	0·50	0·52	0·52	0·52	0·38	0·32	0·35	0·37	0·34	0·36	0·36	0·38
2	0·50	0·52	0·52	0·52	0·23	0·25	0·24	0·27	0·23	0·25	0·27	0·28
4	0·50	0·52	0·52	0·52	0·03	0·07	0·05	0·09	0·03	0·08	0·08	0·12

oxidized ascorbic acid to the same extent as the juice. This would be expected from Szent-Györgyi's observation [1931] that this enzyme in the cabbage does not oxidize phenol. Further this rate of oxidation is found to be uninfluenced by the addition of catechol in both cases. It is, however, of importance to mention in this connexion that only when the oxidation of the ascorbic acid by the juice was complete did that of the catechol begin. This was shown by the development of the red colour, with the subsequent deposition of the dianilinobenzoquinone in the test solution containing catechol and aniline.

The peroxidase activity of cabbage preparations

The cabbage is known to contain a peroxidase and it was desirable to ascertain whether under our experimental conditions this enzyme had any connexion with the oxidative processes described above.

The tests for peroxidase activity of both juice and the solution of the $(NH_4)_2SO_4$ precipitate were made with guaiacol, benzidine, *p*-phenylenediamine and α-naphthol. When 0·1 ml. of 1 % solutions of these reagents was added to 2·0 ml. of juice or enzyme solution negative reactions were obtained, with the possible exception of benzidine, which reagent became slightly coloured only when the juice was used as a source of the enzyme. On the addition of 1 drop of 6 % H_2O_2, however, marked positive reactions were obtained with all reagents in both cases. Tests for the presence of peroxides were carried out on fresh and stored juices and on the precipitated enzyme solution by adding 0·25 ml. 1 % soluble starch solution, 0·25 ml. 5 % KI and 0·5 ml. glacial acetic acid to 2·0 ml. of juice or enzyme. Stored juice alone gave a positive reaction.

Not only did the addition of H_2O_2 enable both preparations to oxidize the above reagents, but it also endowed the juice with the ability to oxidize monohydricphenols and the precipitated enzyme to oxidize both mono- and dihydricphenols, which, as seen above, they were unable to do in its absence.

It would nevertheless seem unlikely that in the absence of a peroxide in the fresh juice ascorbic acid could be oxidized by a coupled reaction involving peroxidase.

THE ENZYMIC OXIDATION OF ASCORBIC ACID BY THE CAULIFLOWER

The juice or the precipitated enzyme, prepared from the frozen florets under the same conditions as above, was used in the following experiments. These were carried out under precisely the same conditions as those with the cabbage.

Similar yields of juice were obtained. In contrast to the cabbage, however, the treatment of the cauliflower juice with alcohol (2 volumes) yielded precipitates of relatively high activity. The $(NH_4)_2SO_4$-precipitated enzyme was, however, used in these experiments.

*p*H *range of activity and sensitivity to cyanide*

As will be seen from Table III, the range and zone of optimum activity of cauliflower juice were similar to those of cabbage juice. The cauliflower juice

Table III. *Activity and sensitivity to cyanide of the cauliflower enzyme*

Time in hours	mg. ascorbic acid in 3·0 ml. of test solution							
	Juice	Juice + *M*/1000 cyanide	Control	Control + *M*/1000 cyanide	Juice	Juice + *M*/1000 cyanide	Control	Control + *M*/1000 cyanide
	Ascorbic acid solutions at *p*H 3·0				Ascorbic acid solutions at *p*H 5·0			
0	0·47	0·47	0·45	0·45	0·45	0·45	0·46	0·46
1	0·45	0·45	0·45	0·45	0·15	0·24	0·45	0·46
1·5	—	—	—	—	0·08	0·18	0·45	0·46
2	0·45	0·45	0·45	0·45	0·02	0·10	0·45	0·46
3	0·45	0·45	0·45	0·45	—	—	—	—
	Ascorbic acid solutions at *p*H 7·0				Ascorbic acid solutions at *p*H 9·0			
0	0·45	0·45	0·45	0·44	0·40	0·40	0·40	0·40
1	0·16	0·35	0·45	0·44	0·30	0·35	0·30	0·30
1·5	0·10	0·30	0·45	0·43	0·24	0·34	0·30	0·30
2	0·04	0·25	0·45	0·44	—	—	—	—
3	—	—	—	—	0·10	0·23	0·30	0·30

appears, however, from this experiment to have been inactive at *p*H 3·0. It is also somewhat more sensitive to *M*/1000 cyanide.

The aerobic nature of the enzyme activity

Like cabbage juice, cauliflower juice was unable to oxidize ascorbic acid under anaerobic conditions in the presence of methylene blue.

Action of the enzyme on mono- and di-hydricphenols

The juice rapidly oxidized catechol but not monohydricphenols (phenol and *m*-cresol) and in the presence of aniline produced dianilinobenzoquinone, thus showing that the process was of the same type as that of the cabbage juice. The $(NH_4)_2SO_4$-precipitated enzyme also resembled that of the cabbage in its inability to cause the oxidation of catechol in air.

Action of the enzyme on ascorbic acid in presence of catechol

The results of this experiment (Table IV) show that catechol did not influence the rate of oxidation of ascorbic acid by the juice or by the $(NH_4)_2SO_4$-precipitated enzyme. As with the cabbage juice, the oxidation of catechol by this juice, as shown by development of red colour and subsequent deposition of red crystalline precipitate in presence of aniline, began only after the oxidation of ascorbic acid was complete. The results are, therefore, analogous to those of the corresponding experiments with cabbage preparations.

Table IV. *Influence of catechol on the activity of the cauliflower enzyme at pH 6·0*

All these tests were made in presence of $M/15$ KH_2PO_4

mg. ascorbic acid in 2·0 ml. of test solution

Time in hours	Control ascorbic acid solutions				Ascorbic acid solutions containing juice				Ascorbic acid solutions containing precipitated enzyme			
		$M/100$ catechol	$M/50$ aniline	$M/100$ catechol and $M/50$ aniline		$M/100$ catechol	$M/50$ aniline	$M/100$ catechol and $M/50$ aniline		$M/100$ catechol	$M/50$ aniline	$M/100$ catechol and $M/50$ aniline
0	0·52	0·51	0·52	0·51	0·48	0·49	0·50	0·50	0·49	0·49	0·49	0·49
1	0·52	0·51	0·52	0·51	0·12	0·14	0·14	0·15	0·07	0·10	0·10	0·11
2	0·52	0·51	0·52	0·51	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00

The peroxidase activity of cauliflower preparations

Tests for peroxidase activity of the juice and the $(NH_4)_2SO_4$-precipitated enzyme were carried out with the same reagents as in the case of the cabbage. In this case too a positive reaction was given by the juice and benzidine only in the absence of H_2O_2. Both the juice and the $(NH_4)_2SO_4$-precipitated enzyme, however, gave very marked peroxidase reactions with all reagents on addition of H_2O_2. Stored, but not fresh, cauliflower juice was found to contain peroxide. In the presence of H_2O_2 the $(NH_4)_2SO_4$-precipitated enzyme and the juice oxidized catechol and *m*-cresol. The peroxidase activity of the cauliflower, therefore, resembles that of the cabbage.

The enzymic oxidation of ascorbic acid by the cucumber

Experiments with cucumber juice and solutions of the enzyme precipitated from it were also carried out under the same conditions as in the case of the cabbage.

Preparation of the juice

The juice, which was invariably at *p*H 5·6, was pressed out of the previously frozen pericarp of the cucumber. Precipitable material was removed by adjusting to *p*H 7·6 and filtering. The juice was then acidified to *p*H 6·0. 80 ml. of juice were obtained from 100 g. of tissue. It was almost colourless and retained its activity on storage at low temperature.

Preparation of the enzyme

The $(NH_4)_2SO_4$ precipitate could not be satisfactorily recovered by centrifuging or by filtration. The enzyme could, however, be precipitated with alcohol or acetone and the latter reagent was chosen for this purpose. The precipitation with acetone was carried out by the method described by Tauber *et al.* [1935], who used Hubbard squash, except that the juice was previously cooled to 0° and that the enzyme was precipitated with alcohol and acetone chilled to −20°. The precipitate from 100 ml. of juice was dissolved in 50 ml. of $M/5$ sodium acetate or $M/15$ KH_2PO_4 (*p*H 6·0). In this case also half of the total activity of the original juice was recovered.

*p*H *range of activity and sensitivity to cyanide*

Table V shows that the juice is practically inactive at *p*H 3·0 and that its optimum range of activity is between *p*H 5·0 and 7·0. It is still very active at

Table V. *Activity and sensitivity to cyanide of the cucumber enzyme*

Time in hours	mg. ascorbic acid in 3·0 ml. of test solution: Juice	Juice + *M*/1000 cyanide	Control	Control + *M*/1000 cyanide	Juice	Juice + *M*/1000 cyanide	Control	Control + *M*/1000 cyanide
	Ascorbic acid solutions at *p*H 3·0				Ascorbic acid solutions at *p*H 5·0			
0	0·51	0·51	0·51	0·50	0·50	0·52	0·50	0·50
1	0·49	0·50	0·49	0·50	0·06	0·51	0·51	0·50
2	0·47	0·50	0·47	0·50	0·00	0·51	0·51	0·50
3	0·45	0·49	0·45	0·50	0·00	0·51	0·51	0·50
	Ascorbic acid solutions at *p*H 7·0				Ascorbic acid solutions at *p*H 9·0			
0	0·50	0·51	0·50	0·50	0·46	0·49	0·43	0·44
1	0·05	0·50	0·50	0·50	0·09	0·43	0·35	0·36
2	0·00	0·50	0·50	0·50	0·02	0·37	0·33	0·33
3	0·00	0·50	0·50	0·50	0·00	0·33	0·31	0·30

*p*H 9·0. *M*/1000 cyanide abolishes its activity at all *p*H values. It thus differs from the corresponding enzyme of the cabbage and the cauliflower in its susceptibility to cyanide.

Aerobic nature of enzymic activity

Cucumber juice was also found unable to oxidize ascorbic acid in the presence of methylene blue under anaerobic conditions.

Action of the enzyme on mono- and di-hydricphenols

The juice of the cucumber resembled that of the cabbage and cauliflower in its ability to oxidize catechol; in the presence of aniline it gave rise to dianilinobenzoquinone. It was inactive towards phenol and *m*-cresol. The acetone precipitate, like the $(NH_4)_2SO_4$ precipitate of the cabbage and cauliflower, had scarcely any effect upon catechol.

Action of the enzyme on ascorbic acid in the presence of catechol

Table VI shows that the rate of oxidation of ascorbic acid by cucumber juice and by the acetone-precipitated enzyme was unaffected by the presence

Table VI. *Influence of catechol on the activity of the cucumber enzyme at* p*H 6·0*

All these tests were made in presence of *M*/15 KH_2PO_4

Time in hours	mg. ascorbic acid in 2·0 ml. of test solution: Control ascorbic acid solutions				Ascorbic acid solutions containing juice				Ascorbic acid solutions containing precipitated enzyme			
		M/100 catechol	*M*/50 aniline	*M*/100 catechol and *M*/50 aniline		*M*/100 catechol	*M*/50 aniline	*M*/100 catechol and *M*/50 aniline		*M*/100 catechol	*M*/50 aniline	*M*/100 catechol and *M*/50 aniline
0	0·52	0·52	0·52	0·52	0·50	0·50	0·50	0·50	0·52	0·51	0·51	0·51
1	0·52	0·52	0·52	0·52	0·10	0·12	0·14	0·12	0·16	0·18	0·18	0·18
2	0·52	0·52	0·52	0·52	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00

of catechol. As in the cases of the two previously mentioned sources, oxidation of catechol by the juice commenced only when that of ascorbic acid was complete.

The peroxidase activity of the cucumber preparations

Strong positive peroxidase reactions were given only in the presence of hydrogen peroxide by the juice and by the solution of the acetone-precipitated enzyme with the reagents used in the previous experiments. Tests for peroxide on both fresh and stored preparations were in this case all negative. On the addition of H_2O_2 the juice was able to oxidize *m*-cresol and the acetone-precipitated enzyme both *m*-cresol and catechol.

The enzymic oxidation of ascorbic acid by the marrow

The small English and Madeira varieties were used. They had colourless pericarps. The juices and the acetone-precipitated enzymes were obtained in the same manner as in the case of the cucumber. $(NH_4)_2SO_4$ and alcohol were found to be unsatisfactory for the precipitation of the enzyme.

pH range of activity and sensitivity to cyanide

It will be seen from Table VII that the marrow juice had a range and zone of optimum activity similar to that of the cucumber. The cyanide-sensitivity is also similar. At *p*H 9·0 the sensitivity to cyanide was found to vary in different preparations.

Table VII. *Activity and sensitivity to cyanide of the marrow enzyme*

Time in hours	mg. ascorbic acid in 3·0 ml. of test solution: Juice	Juice + *M*/1000 cyanide	Control	Control + *M*/1000 cyanide	Juice	Juice + *M*/1000 cyanide	Control	Control + *M*/1000 cyanide
	Ascorbic acid solutions at *p*H 3·0				Ascorbic acid solutions at *p*H 5·0			
0	0·50	0·51	0·50	0·50	0·46	0·49	0·49	0·50
1	0·49	0·51	0·50	0·50	0·14	0·49	0·49	0·50
2	0·48	0·51	0·50	0·50	0·00	0·49	0·49	0·50
3	0·47	0·50	0·50	0·50	0·00	0·49	0·49	0·50
	Ascorbic acid solutions at *p*H 7·0				Ascorbic acid solutions at *p*H 9·0			
0	0·49	0·51	0·50	0·50	0·47	0·47	0·43	0·43
1	0·23	0·50	0·50	0·50	0·30	0·32	0·35	0·35
2	0·08	0·50	0·50	0·50	0·24	0·24	0·32	0·33
3	0·00	0·49	0·50	0·50	0·17	0·17	0·30	0·30

Aerobic nature of enzymic activity

As in all the previous cases marrow juice was unable to oxidize ascorbic acid under anaerobic conditions in the presence of methylene blue.

Action of the enzyme on mono- and di-hydricphenols

Marrow juice and the precipitated enzyme behaved in the same manner as the corresponding cucumber preparations towards phenol and catechol.

Action of the enzyme on ascorbic acid in the presence of catechol

The similarity in properties between the marrow and cucumber enzymes was again apparent in this experiment. Table VIII shows that the addition of

Table VIII. *Influence of catechol on the activity of the marrow enzyme at* p*H 6·0*

All these tests were made in presence of $M/15\,KH_2PO_4$

mg. ascorbic acid in 2·0 ml. of test solution

Time in hours	Control ascorbic acid solutions				Ascorbic acid solutions containing juice				Ascorbic acid solutions containing precipitated enzyme			
		M/100 catechol	*M*/50 aniline	*M*/100 catechol and *M*/50 aniline		*M*/100 catechol	*M*/50 aniline	*M*/100 catechol and *M*/50 aniline		*M*/100 catechol	*M*/50 aniline	*M*/100 catechol and *M*/50 aniline
0	0·58	0·58	0·59	0·58	0·58	0·58	0·58	0·58	0·58	0·58	0·57	0·58
0·5	0·58	0·58	0·58	0·58	0·23	0·23	0·22	0·20	0·32	0·32	0·31	0·34
1·0	0·58	0·58	0·58	0·58	0·13	0·13	0·12	0·12	0·22	0·22	0·23	0·24
1·5	0·58	0·58	0·58	0·58	0·00	0·02	0·00	0·00	0·08	0·08	0·08	0·10
2·25	0·58	0·58	0·58	0·58	0·00	0·00	0·00	0·00	0·03	0·03	0·03	0·03

catechol had no influence upon the rate at which either the juice or the precipitated enzyme oxidized ascorbic acid. This juice also oxidized the catechol immediately after the oxidation of ascorbic acid was complete. As in the previous cases, the precipitated enzyme was without action upon catechol.

Peroxidase activity of marrow preparations

The peroxidase reactions of marrow juice and the precipitated enzyme were similar to those of the cucumber. The activity of the precipitated enzyme was, however, weaker than that of the corresponding cucumber preparation. Neither preparation gave peroxide reactions.

The enzymic oxidation of ascorbic acid by the apple

In the original investigation [Zilva, 1934] crude apple juice was used as the source of the enzyme. As will be seen later the crude juice is most probably active only by virtue of the suspended material with which the enzyme is associated; the pure juice itself appears to be inactive. It will be further seen that the mechanism involved in the oxidation of ascorbic acid by the apple is different from that of the sources described above.

Bramley's Seedling apples were used in the following experiments which were carried out in a similar manner to those previously described.

Preparation of the juices

(*a*) *Crude juice.* The apple after removal of the peel and core was ground with clean sand, squeezed through muslin and the juice centrifuged. The latter was brown in colour and usually at *p*H 3·0.

(*b*) *Pure juice.* The juice was expressed from the previously frozen unpeeled apple, after removal of the core, and filtered. It was invariably colourless and at *p*H 3·0, but on neutralization became orange-brown above *p*H 6·0. This colour disappeared only on re-acidifying immediately. 80 ml. of the juice were obtained from 100 g. of tissue.

Extraction of the enzyme from the juice-free tissue

The tissue residue left after pressing out the juice was separated from the peel, ground with sand and extracted by further grinding with sand under $M/5$ sodium acetate (pH 6·0) or $M/5$ acetic acid (pH 3·0) according to the nature of the experiment. It was then centrifuged and filtered, yielding a brown-coloured solution. 40 ml. of the extracting solution were used to extract the residue from 50 g. of apple.

Activity and cyanide-sensitivity of crude juice, pure juice and extract of juice-free tissue

In the preliminary experiments, owing to the wide variation in activity and the small size of the individual apples, the various facts concerning the nature of the enzymic activity were studied in separate fruits. Later, however, when larger apples became available, the various preparations were obtained from the same fruit whereby a stricter comparison of the results could be obtained. The activity of the respective preparations and their sensitivity to cyanide were investigated at the natural acidity of the apple, pH 3·0, and also at pH 6·0, which was found to be the optimum pH for the oxidation in the above sources. The experiments were carried out under conditions identical with those observed for the cabbage etc. Table IX shows that the crude juice was more active at pH 6·0 than at pH 3·0. At the latter reaction cyanide had no inhibiting effect. In the original investigation one of us had already observed that at pH 4·4 this activity was retarded only to a small extent in the presence of $M/500$ cyanide. The present experiments show, however, that at pH 6·0 the inhibition is very marked. The pure juice or the extract of the juice-free tissue alone had practically no activity at either pH 3·0 or 6·0. Together, however, they were active, especially at pH 6·0 and this activity was inhibited by cyanide to the same extent as in the crude juice. It must be added that with some specimens of the fruit the combined action of pure juice and tissue extract at pH 3·0 was found to be more marked than in the present case.

Action of the enzyme on mono- and di-hydricphenols

It is well known that apple pulp contains a polyphenolase. Experiments were performed in order to ascertain whether the enzyme was present in the tissue or in the pure juice. It was found that the former preparation but not the pure juice oxidized both phenol and catechol directly in the air. In the presence of aniline the characteristic red crystalline precipitate of dianilinobenzoquinone was obtained. This enzyme, therefore, differs essentially from that of the above described plants. In view of this difference and of the fact that the enzyme alone does not oxidize ascorbic acid, it was of interest to study the behaviour of the tissue extract on ascorbic acid in the presence of catechol.

Action of the enzyme on ascorbic acid in the presence of catechol

The results of this experiment (Table X) show that although the extract of the tissue was by itself unable to oxidize ascorbic acid (Table IX), the addition of catechol enables it to do so at a rate approaching that of the crude juice, particularly at pH 6·0. On the other hand, as would have been expected from the preceding experiment, the addition of catechol to the pure juice does not enable it to oxidize ascorbic acid. It would therefore seem that the enzyme is contained in the tissue and that it dehydrogenates ascorbic acid indirectly by

Table IX. *Activity and sensitivity to cyanide of the apple enzyme*

Time in hours	mg. ascorbic acid in 2·0 ml. of test solution: Control solution	Crude juice	Pure juice	Juice-free tissue extract	Juice-free tissue extract + pure juice	*M*/1000 cyanide: Control solution	Crude juice	Pure juice	Juice-free tissue extract	Juice-free tissue extract + pure juice
	Ascorbic acid solutions at *p*H 3·0									
0	0·45	0·45	0·50	0·45	0·52	0·47	0·46	0·47	0·50	0·53
1	0·45	0·35	0·50	0·45	0·50	0·47	0·41	0·47	0·50	0·52
2	0·45	0·30	0·50	0·45	0·47	0·47	0·33	0·47	0·48	0·50
3	0·45	0·25	0·50	0·43	0·46	0·47	0·27	0·47	0·48	0·50
4	0·45	0·20	0·50	0·41	0·46	0·47	0·22	0·47	0·48	0·50
	Ascorbic acid solutions at *p*H 6·0									
0	0·45	0·45	0·50	0·45	0·45	0·45	0·45	0·50	0·45	0·50
1	0·45	0·30	0·50	0·45	0·30	0·45	0·35	0·50	0·45	0·37
2	0·45	0·15	0·50	0·45	0·22	0·45	0·32	0·48	0·45	0·30
3	0·45	0·10	0·50	0·45	0·15	0·45	0·28	0·48	0·45	0·24
4	0·45	0·05	0·48	0·45	0·09	0·45	0·22	0·47	0·45	0·20

Table X. *Influence of catechol on the activity of the apple enzyme*

Time in hours	mg. ascorbic acid in 2·0 ml. of test solution: Ascorbic acid solutions at *p*H 3·0: Control solution	Crude juice	Pure juice + *M*/100 catechol	Juice-free tissue extract + *M*/100 catechol	Ascorbic acid solutions at *p*H 6·0: Control solution	Crude juice	Pure juice + *M*/100 catechol	Juice-free tissue extract + *M*/100 catechol
0	0·45	0·45	0·50	0·45	0·45	0·45	0·50	0·45
1	0·45	0·35	0·50	0·40	0·45	0·30	0·50	0·30
2	0·45	0·30	0·50	0·38	0·45	0·15	0·48	0·17
3	0·45	0·25	0·50	0·34	0·45	0·10	0·48	0·12
4	0·45	0·20	0·50	0·30	0·45	0·05	0·48	0·03

previously oxidizing a soluble compound, probably a phenolic substance, present in the juice. The enzyme system involved in the oxidation of ascorbic acid in the apple preparations is thus distinct from those present in the previously described plants and resembles that described by Szent-Györgyi in the case of the potato [1931]. That this enzyme is more or less evenly distributed in all the tissue of the receptacle of the apple can be seen from the fact that when a solution of catechol and aniline is spread over the cut surface of a medial section of the fruit a uniform red colour develops immediately over the entire surface.

The enzymic oxidation of ascorbic acid by the potato

The King Edward variety was used in these experiments which were carried out under the conditions already described.

Preparation of the juice

The juice was expressed from the previously frozen tubers. It was invariably at *p*H 6·0. The whole juice, which was free from suspended matter, darkened on standing and deposited a black precipitate.

Preparation of the enzyme

The enzyme was precipitated from the fresh juice with 4 volumes of 96 % alcohol and washed with 80 % alcohol at the centrifuge. It was dissolved in a volume of *M*/5 sodium acetate (*p*H 6·0) equal to that of the original juice. In some preparations the enzyme was reprecipitated. The filtered enzyme solution was at first yellow in colour but darkened during the first few minutes without, however, depositing any precipitate later. Acetone and $(NH_4)_2SO_4$ precipitations were not attempted in this case.

Activity and cyanide-sensitivity of the juice and precipitated enzyme

It will be seen from Table XI that at *p*H 6·0 the precipitated and reprecipitated enzymes oxidized a certain amount of the ascorbic acid during the first hour, after which time oxidation practically stopped. The juice on the other hand oxidized ascorbic acid rapidly at *p*H 6·0 (Tables XI and XII). From

Table XI. *Influence of catechol on the activity of the potato enzyme*

All these tests were made in presence of *M*/5 sodium acetate

mg. ascorbic acid in 2·0 ml. of test solution

	Ascorbic acid solutions containing juice				Ascorbic acid solutions containing precipitated enzyme				Ascorbic acid solutions containing reprecipitated enzyme			
Time in hours		*M*/100 catechol	*M*/50 aniline	*M*/100 catechol and *M*/50 aniline		*M*/100 catechol	*M*/50 aniline	*M*/100 catechol and *M*/50 aniline		*M*/100 catechol	*M*/50 aniline	*M*/100 catechol and *M*/50 aniline
0	0·50	0·50	0·50	0·50	0·49	0·49	0·50	0·50	0·92	0·92	0·92	0·92
1	0·30	0·03	0·30	0·00	0·40	0·06	0·39	0·04	0·82	0·55	0·87	0·58
2	0·16	0·00	0·22	—	0·35	0·00	0·35	0·00	0·80	0·30	0·83	0·37
3	0·10	—	0·15	—	—	—	—	—	—	—	—	—
4	—	—	—	—	0·35	—	0·35	—	—	0·05	—	0·05
5	—	—	—	—	—	—	—	—	0·75	—	0·75	—

Table XII. *Activity and sensitivity to cyanide of the potato enzyme*

mg. ascorbic acid in 2·0 ml. of test solution

	Ascorbic acid solutions at *p*H 3·0						Ascorbic acid solutions at *p*H 6·0					
				M/1000 cyanide						*M*/1000 cyanide		
Time in hours	Control solution	Juice	Juice + *M*/100 catechol	Control solution	Juice	Juice + *M*/100 catechol	Control solution	Juice	Juice + *M*/100 catechol	Control solution	Juice	Juice + *M*/100 catechol
0	0·54	0·54	0·54	0·54	0·54	0·54	0·52	0·51	0·50	0·55	0·51	0·49
1	0·54	0·53	0·53	0·54	0·54	0·53	0·52	0·30	0·20	0·55	0·38	0·34
2	0·53	0·52	0·52	0·54	0·52	0·52	0·52	0·20	0·11	0·54	0·30	0·19
3	0·52	0·50	0·50	0·54	0·51	0·50	0·52	0·16	0·00	0·54	0·21	0·06

Table XII it is also seen that cyanide inhibited its activity but to a small extent and that at *p*H 3·0 the juice showed no activity at all. Sufficient experiments were not performed with the potato to ascertain whether the activity at this *p*H varies in individual cases as it does with the apple.

Action of the enzyme on mono- and di-hydricphenols

The potato juice and the precipitated enzyme, as would have been expected from the literature, oxidized both phenol and catechol directly in the air. In the presence of aniline the red dianilinobenzoquinone was formed.

Action of the enzyme on ascorbic acid in the presence of catechol

It will be seen from Tables XI and XII that the addition of catechol to the juice enhances its activity at *p*H 6·0 but does not alter its behaviour at *p*H 3·0. Table XI shows that in the presence of catechol the precipitated enzyme acquires the capacity of dehydrogenating ascorbic acid. These results are similar to those already described by Szent-Györgyi [1931]. The ascorbic acid-oxidizing system in the potato, therefore, resembles that of the apple. The oxidizing enzyme appears to be restricted to a peripheral layer which can be shown by the method adopted in demonstrating the distribution of the enzyme in the apple. This last observation is in agreement with the findings of Wieland & Sutter [1930].

SPECIFICITY OF THE ASCORBIC ACID-OXIDIZING ENZYME

As has been mentioned already, the aerobic oxidase of the apple was previously found to be able to oxidize *d*-gluco-ascorbic acid, which is antiscorbutically inactive. From the experiments described above it now seems that this oxidation is not direct but is due to the action of compounds resulting from the enzymic oxidation of substances, probably polyhydricphenols, present in the juice. Since this mechanism is different from that of the cabbage, cauliflower, cucumber and marrow, which oxidize ascorbic acid directly, it was of interest to test the action of the enzyme of the latter group upon the above analogue of ascorbic acid. The precipitated enzyme of the marrow was used as a representative of this group of enzymes. Its effect upon *d*-gluco-ascorbic acid is given in Table XIII and it is seen that this enzyme also rapidly oxidizes this substance.

Table XIII. *Oxidation of* d-*gluco-ascorbic acid by the marrow enzyme*

	mg. *d*-gluco-ascorbic acid in 2·0 ml. of test solution	
Time in hours	*d*-Gluco-ascorbic acid in *M*/15 KH_2PO_4 *p*H 6·0	*d*-Gluco-ascorbic acid and precipitated marrow juice enzyme in *M*/15 KH_2PO_4 *p*H 6·0
0	0·70	0·70
0·5	0·70	0·35
1·0	0·70	0·23
1·5	0·70	0·14
2·0	0·70	0·05
2·5	0·70	0·00

DISCUSSION OF RESULTS

The results are summarized in Table XIV. The principal point that emerges from this investigation is that among the plants examined ascorbic acid is dehydrogenated by two different mechanisms. One is characteristic of the cabbage, cauliflower, cucumber and marrow, the other of the apple and the potato. The former group contains an aerobic oxidase which although unable to act on phenols can oxidize ascorbic acid directly. The latter group, on the other hand, does not contain such a specific enzyme but can dehydrogenate ascorbic

Table XIV

Source of enzyme: Plant	Source of enzyme: Part	*p*H activity: Range of *p*H	*p*H activity: Optimum *p*H	Inhibiting effect of NaCN *M*/1000	Action on ascorbic acid	Action on catechol	Action on ascorbic acid in presence of catechol	Remarks
Cabbage	Leaves after removal of central ribs. Enzyme precipitated from juice by $(NH_4)_2SO_4$	3·0–9·0. More active at alkaline side of optimum *p*H	5·0–7·0	Not complete, greatest at neutrality	Juice and enzyme* oxidize it directly	Juice but not enzyme oxidizes it. In presence of aniline, dianilinobenzoquinone formed by the juice but not by the enzyme	Oxidation of catechol begins only after ascorbic acid is completely dehydrogenated	Preparations of enzyme uniform in stability and activity. Juice and enzyme contain peroxidase. Peroxides found in stored juice. Both preparations oxidize phenol only in presence of H_2O_2
Cauliflower	Florets. Enzyme precipitated from juice by $(NH_4)_2SO_4$	Not active at 3·0. Found active at 5·0–9·0	Similar to cabbage	Similar to cabbage	Similar to cabbage	Similar to cabbage	Similar to cabbage	Similar to cabbage
Cucumber	Pericarp. Enzyme precipitated from juice by acetone	Similar to cauliflower	Similar to cabbage	Complete at all *p*H values	Similar to cabbage	Similar to cabbage	Similar to cabbage	Similar to cabbage except no peroxide found after 2 weeks' storage
Marrow	Similar to cucumber	Similar to cauliflower and cucumber	Similar to cabbage	Similar to cucumber	Similar to cabbage	Similar to cabbage	Similar to cabbage	Similar to cabbage except no peroxide found after 2 weeks' storage
Apple	Receptacle of fruit from which were prepared (*a*) crude juice by direct expression, (*b*) pure juice by expression of previously frozen fruit, (*c*) tissue extract by treatment of juice-free tissue with buffer	3·0–8·0	6·0	Partial at 6·0. Not so great as in case of cabbage. Little at 3·0	Pure juice and tissue extract alone do not oxidize it but are able to do so when combined. Crude juice which contains tissue debris oxidizes it	Oxidizes it. In presence of aniline dianilinobenzoquinone is formed by crude juice and tissue extract but not by pure juice	Tissue extract and crude, but not pure, juice oxidize it	Stable for considerable period when stored in cold at *p*H 6·0. Activity of preparations not so consistent as cabbage; and that of the tissue extracts and some crude juices very low at *p*H 3·0. Crude juice and tissue extract oxidize phenol and catechol directly. Both contain peroxidase
Potato	Tuber. Enzyme precipitated from juice by alcohol	Tested at 3·0 and 6·0. Only active at 6·0	Not ascertained	Not very marked	Juice oxidizes it. Enzyme small initial oxidation only	Both juice and enzyme oxidize it	Enzyme oxidizes it. Activity of juice enhanced	Juice and enzyme similar to crude juice and tissue extracts of apples respectively

* In all cases the term enzyme refers to the precipitated enzyme.

acid indirectly by previously oxidizing mono- and poly-hydricphenols, if these are present in the medium, to their corresponding quinones, which in their turn oxidize ascorbic acid. The juice of the cabbage group of plants besides possessing a direct ascorbic acid oxidase can also oxidize catechol directly, but this reaction, unlike that of the apple and the potato, begins only when the oxidation of the ascorbic acid present is complete.

The direct dehydrogenation of ascorbic acid by some of the above plants, cabbage etc., seems to be due to one enzyme. Although the sensitivity of the reaction to cyanide differs somewhat with the source, this fact is not sufficient in itself to justify the assumption that different oxidases are involved. It is known that the same enzyme but of different origins when acting on the same substrate can be inhibited by cyanide to different extents. Phenolase may be mentioned in this connexion [cf. Suminokura, 1930; Wieland & Sutter, 1928; 1930]. Nor can the different suitabilities of the reagents in the precipitation from the various sources be taken as an indication that the enzyme is not identical in each case, since the variation of the amount of inactive precipitable material would determine the choice of the most appropriate reagent.

The tissues of a great number of plants can, therefore, oxidize ascorbic acid *in vitro* by any one of at least three different enzyme systems, namely indirectly by peroxidase [Szent-Györgyi, 1928] and, as seen above, by phenolases such as are present in the apple and the potato and directly by an aerobic oxidase contained in the cabbage, etc.[1] This last enzyme has been further found to be capable of dehydrogenating another member of the ascorbic acid series of compounds. Whether the specificity of this oxidase is restricted only to this category of substances cannot at present be answered. Preliminary experiments with reductone and dihydroxymaleic acid, which reduce indophenol, although suggestive that these substrates may be oxidized by the enzyme, were complicated by other factors and consequently did not yield results justifying a final conclusion.

The results so far obtained with disrupted tissues *in vitro* leave their significance in the biological processes of the plant a matter for speculation. Constructive suggestions cannot reasonably be made until information bearing on the point is obtained in the living plant before the disintegration of the tissues. Some information of this character has already been obtained. Thus indications were found that in the apple part of its vitamin C is present as dehydroascorbic acid in the living tissue [Zilva *et al.* 1935]. The equilibrium between ascorbic acid and its dehydrogenated form in the living cell has since been followed up by these workers during growth, maturity and senescence of the apple. The results of this investigation will form the subject of another communication.

[1] The plant differs in this respect from the animal since the disrupted tissues of the latter do not oxidize ascorbic acid. I should like to take this opportunity of correcting an error in a previous publication [Kellie & Zilva, 1936]. In Table IV (p. 365), "Corpuscles + 1·5 mg. ascorbic acid/100 ml." and "Whole blood + 1·5 mg. ascorbic acid/100 ml. blood" should read "Trichloroacetic acid extract of corpuscles" etc. and of "whole blood etc." respectively. This control was performed in order to demonstrate that genuine ascorbic acid, unlike the indophenol-reducing substance of these extracts found after treatment with H_2S, was not affected by the passage of a current of nitrogen. Borsook *et al.* [1937, p. 237] are undoubtedly right in asserting that ascorbic acid added *before* precipitation with trichloroacetic acid is oxidized to dehydroascorbic acid at least to some extent. This would be expected from our work on the influence of laked blood on ascorbic acid. (S. S. Z.)

SUMMARY

The cabbage, cauliflower, cucumber and marrow contain an enzyme which oxidizes *l*-ascorbic acid and *d*-gluco-ascorbic acid directly. This enzyme does not oxidize mono- or di-hydricphenols.

The juices of these plants oxidize catechol as well as ascorbic acid. When both are present the oxidation of the former begins only after the oxidation of the latter stops.

No enzyme capable of oxidizing *l*-ascorbic acid directly has been found in the apple or the potato. The phenolases present in these plants can, however, oxidize it in the presence of catechol or of the juice. This ascorbic acid-oxidizing system is therefore distinct from that of the cabbage, cauliflower, cucumber and marrow.

The presence of peroxidase, but not of peroxide, could be established in the fresh juices of the cabbage, cauliflower, cucumber and marrow.

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